THE ROLE OF TLR7 IN MEDIATING SEX DIFFERENCES IN RESPONSE TO INFLUENZA INFECTION AND VACCINATION

by

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ABSTRACT

Among adult humans, females reportedly have more severe morbidity than males following exposure to novel strains of influenza A viruses (e.g., pandemic or avian influenza viruses). Females also have higher antibody titers to influenza vaccine antigens than their male counterparts. Male-female differences in influenza virus infection and vaccine responses can be recapitulated in mice suggesting that biological differences between the sexes may be involved. Toll-like receptor 7 (TLR7) is a pattern recognition receptor for single stranded RNA viruses and plays a role in the innate detection and activation of inflammatory signaling in plasmacytoid dendritic cells (pDCs). TLR7 also exists in B cells and signaling in B cells is important for antibody response, including isotype switching. TLR7 is located on the X chromosome and was recently shown to escape X inactivation in B cells. Whether females have greater dependence than males on TLR7-dependent signaling during influenza virus infection or vaccination has not been reported and was tested in the current thesis. Adult male and female wild-type and *Tlr7* deficient mice on a C57BL/6NJ background were infected with an influenza A virus (i.e., mouse adapted A/CA/09 H1N1 [2009 H1N1]) and monitored for virus replication kinetics, pulmonary inflammation, and morbidity to characterize possible sex differences in influenza pathogenesis in the absence of TLR7-depedent signaling. Among wild-type mice, females experienced greater morbidity, cleared virus faster, but had similar levels of pulmonary inflammation

as compared with males. Female-biased morbidity, but not virus clearance, still occurred among *Tlr7* deficient mice, suggesting that elimination of TLR7-depedent signaling did not alter sex differences in the pathogenesis of influenza. Next adult male and female wild-type and *Tlr7* deficient mice received an inactivated 2009 H1N1 vaccine using a prime-boost strategy. Serum samples were collected at several time-points to evaluate antibody responses, and animals were challenged with a 2009 H1N1 drift variant virus to evaluate protection following vaccination. Among wild-type mice, females produced greater neutralizing, total IgG, and IgG2c titers that had a greater avidity index than males. In the absence of *Tlr7*, the sex difference in titers of neutralizing antibody, total IgG antibody, and IgG2c antibody titers as well as the avidity index following vaccination were eliminated, primarily due to a significant decline in antibody responses from *Tlr7*$^{-/-}$ females. Following virus challenge, wild-type females cleared virus from their lungs faster than males and this female-biased control of virus replication was eliminated in the absence of *Tlr7*. Taken together our results demonstrate that *Tlr7* promotes a more robust vaccine-induced antibody response in females and the absence of *Tlr7* eliminates the sex-specific difference in viral clearance following challenge. Influenza vaccination reportedly results in sex-specific antibody responses following vaccination and our data suggests this may be due to TLR7. Greater consideration should be given to sex-dependent mechanisms mediating vaccine-induced immune responses and protection.

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2009 H1N1	Mouse adapted A/CA/09 H1N1
APC	Antigen-presenting cell
CPE	Cytopathic effect
DC	Dendritic cell
dpc	Days post-challenge
dpi	Days post-inoculation
dpv	Days post-vaccination
H&E	Hematoxylin and eosin
HAI	Hemagglutination
HCl	Hydrochloric acid
IAV	Influenza A virus
IFN	Type I interferons
IL-6	Interleukin-6
im	Intramuscular
in	Intranasal
ip	Intraperitoneal
IRF	Interferon regulatory factor
ISG	IFN-stimulated genes
L.O.D.	Limit of detection
ma2009dv	Mouse-adapted A/California/04/09 drift variant virus
MAPK	Mitogen-activated protein kinase
MAVs	Mitochondrial antiviral signaling
MyD88	Myeloid-differentiation primary response gene88
NF-κB	Nuclear factor κB
NLRP3	NOD-like receptor family member NOD-, LRR- and pyrin domain-containing 3
OD	Optical density

PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cells
pdm	Pandemic
PRR	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene I
TIV	Seasonal influenza virus trivalent inactivated vaccine
TLR	Toll-like receptors
TLR7	Toll-like receptor 7 protein
Tlr7	Toll-like receptor 7 gene in mice
TNF-α	Tumor necrosis factor-α
TRIF	TIR-domain-containing adapter-inducing interferon-β
vRNA	Viral RNA
XIC	X chromosome inactivation

INTRODUCTION

Host Recognition of Viruses

I. Toll-like Receptors

Toll-like receptors (TLR) are arguably the most fundamental immune receptors in our body, in spite of their recent discovery in 1996 (Lemaitre, Nicolas, Michaut, Reichhart, & Hoffmann, 1996). As a core part of our inherited resistance to disease, TLR proteins allow mammals to detect invading microbes and initiate an appropriate response (Beutler, 2004). Most of the phenomena that occur in the course of infection are orchestrated by TLRs or other pattern recognition receptors (PRRs) found on or inside our cells (Beutler, 2004). Despite the extreme chemical complexity of microbial stimuli, only a handful of TLRs and two adaptor proteins are needed for the detection of most microbes (Beutler, 2004). This is possible because each TLR recognizes a different evolutionarily conserved pathogen-associated molecular pattern (PAMP) that is both semi-specific to the microbial world and invariant among entire classes of pathogens (Iwasaki & Medzhitov, 2004; Janeway Jr., 1998). All together the TLRs recognize the molecular patterns of almost all bacteria, fungi, and viruses that infect mammals, including humans(Murphy, Travers, Walport, & Janeway, 2012).

Although the of ratio of TLRs to microbial molecules is extremely small (10:>1,000), the secondary/tertiary events triggered by TLR activation are highly specific with a ratio closer to 1:1

(Beutler, 2004). This complexity is conferred through the type of cell expressing the TLR, the level of TLR expression, the combination of PRRs activated, the timing of TLR activation, the hundreds of different effectors responsive to TLR signaling, and recent evidence suggests the sex of the host (Liu & Ding, 2016; Souyris et al., 2018). Following stimulation by microbes, TLRs transduce a signal through adaptor proteins myeloid-differentiation primary response gene88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF). This signal is propagated through serine kinases, ultimately resulting in the activation of a specific set of transcription factors, most notably NF-kB, interferon regulatory factor (IRF) family members, and mitogen-activated protein kinase (MAPK) (Beutler, 2004).

While bacteria, fungi, and parasites are recognized by cell-surface TLRs that target multiple molecules associated with their structure and metabolism, viruses are primary recognized by their nucleic acids alone. Thus, TLRs that recognize nucleic acids play a critical role during viral infection. In humans and mice these TLRs include TLR3 which responds to dsRNA, TLR9 which responds to dsDNA, and TLR7 and TLR8 (humans only) which respond to purine-rich ssRNA. The biological relevance of intracellular TLRs can be inferred from their evolution under strong purifying selection. In human populations, intracellular TLRs have extremely low rates of nonsynonymous and stop mutations not seen among cell-surface TLRs. Worldwide, TLR7 and TLR9 have the least genetic diversity in human populations. This is likely because intracellular TLRs play a specialized, non-redundant role in host survival, either via protective immunity against viral infections (present or past), additional involvement in other non-immunity related important biological processes, or both (Barreiro et al., 2009; Souyris et al., 2018). Understanding the role of TLRs in mediating the immune response to viruses, such as influenza A viruses (IAVs), particularly in the context of vaccination, therefore continues to be an important area of study and will be the focus of this thesis.

II. TLR7, Plasmacytoid Dendritic Cells, and Type I Interferons

During an infection with IAV, TLR7 is one of multiple PRRs used to recognize unique features associated with the virus. PRRs that recognize influenza viruses can be organized into three distinct

families: 1) the TLRs, including TLR3, TLR7, and TLR8; 2) the retinoic acid-inducible gene I (RIG-I); and 3) the NOD-like receptor family member NOD-, LRR- and pyrin domain-containing 3 (NLRP3) (Iwasaki & Pillai, 2014; Pang & Iwasaki, 2012). They can also be categorized by whether their viral sensing is cell-intrinsic, mediated by infected cells, or cell-extrinsic, mediated by uninfected cells. RIG-I and NLRP3 sense IAV through cell-intrinsic recognition when viral nucleic acids or viral damage is detected in the cytosol. They are ubiquitously expressed in the cytosol of immune and non-immune cells. Alternatively, TLR3 and TLR7 generally sense IAV through cell-extrinsic recognition when they detect viral nucleic acids in endosomes. These PRRs tend to be expressed in sentinel cells such as macrophages and dendritic cells (DCs) where they are used to sample phagocytosed material for the presence of viral nucleic acids. (Iwasaki & Medzhitov, 2010; Pang & Iwasaki, 2012; Stetson, 2009).

Following the infection of non-immune cells, primarily respiratory epithelial cells, with IAV, RIG-I is activated and following the detection of IAV by sentinel immune cells, TLRs are activated. Activation of TLRs and RIG-I triggers a signaling cascade that culminates in the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) through nuclear factor κB (NF-κB), and the production of Type I interferons (IFN) through IRF3 and/or IRF7 activation. During a viral infection (e.g., with IAV), the predominant effectors are IFNs. IFNs drive the expression of over 300 genes, collectively known as IFN-stimulated genes (ISGs) which cooperate to control virus infection and spread (Katze, M.G., He, Y. & Gale, M., 2002; Lund et al., 2004; Pang & Iwasaki, 2012). Type I IFNS and ISGs induce antiviral protection by establishing an antiviral state in neighboring cells and by activating key cells in the adaptive immune responses for efficient viral clearance (Stetson & Medzhitov, 2006; Takeuchi & Akira, 2007).

The primary producers of type I IFNs are plasmacytoid dendritic cells (pDCs) which produce from a hundred to a thousand times more IFN-α than other cell types (Hoffmann, Schneider, & Rice, 2015; Siegal, 1999). This is thought to be possible because pDCs express high levels of TLR7 (Berghofer et al., 2006; Kadowaki & Liu, 2002). In pDCs, which do not express TLR3 in mice or humans, activation of TLR7 allows for the recruitment of IRF7 and the induction of robust levels of

type I IFNs (Pang & Iwasaki, 2012). TLR7 signaling in pDCs also upregulates cytosolic sensors of viral products, such as RIG-I, further amplifying the induction of the antiviral state (S. S. Diebold, 2004; Sandra S Diebold et al., 2003). One advantage for the immune system of using this strategy against viral infections is that TLR7 is activated in a cell-extrinsic manner and thus not directly inhibited by viral proteins that antagonize PRRs like RIG-I, associated with type I IFN induction in infected cells (Stetson, 2009).

Although the role of TLR7 in pDCs appears to be unique, mouse models have shown that the TLR7 and the RIG-I pathways are redundant during viral infection. Both TLR7 and RIG-I pathways compensate for the loss of the other upon high-dose IAV challenge, for example (Pang, Pillai, & Iwasaki, 2013). A systematic comparison of the genes activated by TLRs and RIG-I-like receptors revealed that a small portion of genes are uniquely turned on by TLRs (Iwasaki & Pillai, 2014; Negishi et al., 2012). Thus, the redundancy between TLR7 and RIG-I in the innate immune response to IAV infection may be specific to the extent of viral burden. Furthermore, while the roles of TLR7 and RIG-I may be redundant in the innate immune response to IAV, their roles in the adaptive immune response which ultimately results in viral clearance and recovery are very distinct. Specifically, antibody responses during IAV mainly rely on TLR7 expressed by B cells (Heer et al., 2007; Jeisy-Scott et al., 2012).

III. TLR7 in B cells

Triggered by the innate immune response during the early phase of IAV infection, the adaptive immune response uses two different effector mechanisms to clear the infection and allow for recovery. These two effector mechanisms are the production of antibodies by B cells which bind and block viral spread, and the activation of cytotoxic cells such as CD8+ T cells which eliminate virus-infected cells. The initiation of both mechanisms is dependent on specific PRRs expressed in antigen-presenting cells (APCs) and B cells. Human and murine B cells express several TLRs; the expression of TLR7 in immune cells at steady state, however, is close to its highest in B cells (Bekeredjian-Ding & Jego, 2009; Hua & Hou, 2013). The detection of non-replicating, single stranded IAV nucleic acid via TLR7 by B cells induces the production of IgG2c antibodies and CD4+ T cell responses (Koyama et al., 2007;

Lopez et al., 2004; Thomas et al., 2009). The production of IgG2c antibodies is critical for lasting immunity and the role of TLR7 in eliciting such responses in B cells has become a major area of focus in vaccine research.

Whole inactivated vaccines such as those used for seasonal IAV vaccination, contain viral genomic RNA and rely heavily on TLR7 engagement to elicit adaptive immunity (Jeisy-Scott et al., 2012). When *Tlr7* deficient mice were immunized with split inactivated IAV, they had significantly reduced antibody titers. Furthermore, following challenge, immunized *Tlr7* deficient mice were less able to control IAV replication, with lung viral titers 100-fold higher than immunized control mice (Jeisy-Scott et al., 2012). Another study obtained similar results, with *Tlr7* deficient mice being unprotected against a lethal dose of IAV following two immunizations with inactivated virus (Koyama et al., 2007). Additionally, an analysis of serum IgG titer (IgG2c, in particular) against "immunodominant" HA Ag was found to be significantly reduced in immunized *Tlr7* deficient mice as compared to immunized controls just prior to challenge.

The mechanism by which TLR7 confers antibody-mediated protection following vaccination appears to be complex and involve both direct and indirect signaling pathways with B cells. An in vitro study of B cells found that TLR7 induces isotype switching indirectly through the activation of T helper cells and pDC which both act on B cells, and directly through TLR7 signaling within B cells (Heer et al., 2007; Koyama et al., 2007). When B cells were stimulated with TLR7 or CD4 T cell ligands directly, they proliferated and produced IgG1, but the production of IgG2c required both of these signals together with pDC production of IFNα (Heer et al., 2007). IgG2c (sometimes referred to as IgG2a) is the major isotype produced during a viral infection and the most efficient in antibody-mediated protection (Coutelier, 1988; Markine-Goriaynoff & Coutelier, 2002; Rubtsova, Rubtsov, van Dyk, Kappler, & Marrack, 2013; Schmitz et al., 2012). In addition to TLR7, T-bet expression in B cells have been shown to be critical for isotype switching to IgG2c during viral infection (Deng et al., 2016; Gerth, Lin, & Peng, 2003; Rubtsova et al., 2013; Snapper & Paul, 1987). Furthermore, an *in vitro* study found the combination of a TLR and BCR agonist induced the highest expression of T-bet in splenic B cells

purified from mice (Rubtsova et al., 2013). Together these data suggest that TLR7 in cells of the innate and adaptive immune system may work together synergistically to induce class-switching to IgG2c in response to viral infection.

Sex influences innate and adaptive immune responses

In addition to its function in B cells, TLR7 is also unique in its location on the X chromosome (Christensen et al., 2006). Although the mechanism of X chromosome inactivation (XIC) in placental mammals was considered extremely stable in adults, recent studies have shown some genes on the X chromosome that are outside the pseudoautosomal regions shared with the Y chromosome, escape XIC. Studies have observed 15% of X-chromosomal genes in human primary fibroblasts and 3.3% in mice kidney cells, escape X inactivation (Carrel & Willard, 2005; Yang et al., 2010). Most recently a study demonstrated that TLR7 is transcribed on both X chromosomes in a large proportion of the pDCs, B cells, and monocytes from normal women. The researchers also found that in B cells this biallelic expression of TLR7 led to increased TLR7 gene products and enhanced B cell responses to TLR7 engagement (Souyris et al., 2018). Our lab has been able to replicate these results in the context of vaccinated C57BL/6 mice with female B cells expressing higher levels of TLR7 mRNA 28 days following vaccination as compared to vaccinated males (Dr. Ashley Fink, unpublished data).

The recent observation of sex differences in TLR7 expression by immune cells is coupled with an increasing number of sex difference observations in viral disease pathogenesis and vaccine response among humans. Generally human studies have shown that females tend to generate a stronger innate and adaptive immune response compared to males. Depending on whether a strong immune response is beneficial or damaging for host survival, females suffer more or less than men (Klein & Roberts, 2015). In the context of influenza infection, females of reproductive age have been shown to suffer more than their male counterparts. During the 2009 H1N1 pandemic (pdm), females were more likely than males to develop severe disease. Interestingly the male-female differences in incidence, severity and mortality rates of 2009 H1N1pdm infection varied by age at infection (CDC, n.d.; Peretz, Hall, & Klein, 2015). Regardless of hospitalization status, males less than 19 years of age had a higher risk of

laboratory confirmed H1N1pdm than females. But, among adults aged 18-64 years, both hospitalized and not hospitalized, women had a higher risk of laboratory confirmed H1N1pdm (Jacobs et al., 2012; Peretz et al., 2015).

In addition to influenza pathogenesis, sex differences have also been observed in response to influenza vaccination among humans. Following receipt of seasonal influenza virus trivalent inactivated vaccine (TIV), women have a higher hemagglutination (HAI) antibody titer than males (Peretz et al., 2015). This trend is seen with different assays, across multiple IAV vaccine strains, and in both young (18-64 years of age) and older (65+ years of age) females. Adult human females given a half dose of TIV had an equivalent or higher neutralizing antibody response compared to adult men given a full dose (Engler, 2008). Even when comparing males and females of the same age, females had a greater absolute change in neutralizing antibody titers against H3N2 and influenza B antigens following TIV than males (Furman et al., 2014; Peretz et al., 2015). This increased antibody response in females was also associated with higher levels of proinflammatory molecules and more adverse reactions following vaccination (Furman et al., 2014).

IV. Animal models of influenza infection and vaccination

Understanding the cause and effect of sex differences in the human population is difficult for ethical reasons, however animal models offer a unique opportunity for expanding our knowledge when sex is considered as a biological variable. Unfortunately, many preclinical studies only use one sex and/or do not state the sex of their animals (Potluri, Engle, Fink, vom Steeg, & Klein, 2018). This has had severe consequences on clinical trial participants and more generally on our ability to leverage beneficial sex differences to develop new treatments.

When sex has been considered as a biological variable in the context of influenza infection, mouse models have enabled researchers to understand how sex differences in the immune response lead to more severe disease in females. Specifically, mouse models have shown female mice have an increased inflammatory response to infection and that this leads to increased pathology. Within the first week following infection with H1N1, female C57/BL6 mice show a greater induction of cytokines

and chemokines in their lungs than males (Peretz, Hall, & Klein, 2015). Additionally, this trend has been seen across different virus and mouse strains. When adult BALB/c mice are infected with H3N1 IAV, females develop greater lung hyperresponsiveness to methacholine challenge and produce more CCL2, an inflammatory cytokine, than males (Boylen, Sly, Zosky, & Larcombe, 2011; Peretz et al., 2015).

Animal models have also provided new insight into the differential efficacy of vaccines in each sex. Similar to humans, following vaccination with ma H1N1 or H3N2, female mice of reproductive ages mount higher neutralizing and total antibody responses than males (Lorenzo et al., 2011; Peretz et al., 2015). Furthermore when immunized mice are challenged with a lethal dose of a different IAV strain, males experience greater morbidity and have higher viral titers in their lungs (Lorenzo et al., 2011). In response to vaccines female mice mount higher anti-IAV antibodies and have better cross protection against IAV (Lorenzo et al., 2011). Interestingly, both male and female mice were equally protected when challenged with a lethal dose of the same virus against which they were immunized, suggesting sex differences in the quality of antibody are complex and require more research.

Hypotheses and aims

Despite strong evidence of sex differences in influenza vacation and the emerging role of TLR7 in the immune response to vaccination, TLR7 has yet to be studied in the context of sex differences in response to influenza infection or vaccination. Therefore, the primary aim of this study is to fill this critical knowledge gap and support further research in this area. Because TLR7 is encoded on the X chromosome (Christensen et al., 2006), has been associated with sex-specific immune responses to viruses (Heil et al., 2004; Souyris et al., 2018) and autoimmune diseases (Christensen et al., 2006; Deane et al., 2007), I hypothesized that deletion of TLR7 will improve the outcome of IAV infection in females to a greater extent than males. The second part of my project will explore sex differences in response to IAV vaccination a subsequent challenge. Because females have higher antibody responses, including IgG and respective isotypes than males, which may be mediated by signaling through TLR7

in B cells, I hypothesize that deletion of TLR7 will reduce antibody responses and the efficacy of the killed IAV vaccine to a greater extent in females than males.

METHODS

Ethics statement.

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee under animal protocol M015H236.

Mice.

Adult (age, 7 to 8 weeks) male and female C57BL/6NJ mice were obtained from Jackson Laboratory. Before handling, animals were kept in the facility for at least 1 week to acclimate. *Tlr7*$^{-/-}$ and *Tlr7*$^{-/y}$ mice (B6.129S1Tlr7tm1Flv/J) on C57BL/6NJ background were a gift from Dr. Patricia Gearhart (National Institute of Aging) and bred at the Johns Hopkins Bloomberg School of Public Health. All animals were housed at up to 5 mice per microisolator cage under standard biosafety level 2 housing conditions, with food and water provided *ad libitum*. Mice were identified via ear punch.

Influenza A viruses and vaccine.

Mouse-adapted influenza A virus A/California/04/09 (ma2009; H1N1) generated by Dr. Andrew Pekosz from a published sequence (Ye et al., 2010) was used for virus infection. A mouse-adapted A/California/04/09 drift variant virus (ma2009dv; H1N1) containing the K166Q mutation of the HA sequence was generated by Dr. Ashley Fink in our lab by reverse genetics and used for virus challenge.

Infectious virus titers of both viruses were determined using the 50% tissue culture infective dose ($TCID_{50}$) assay.

The inactivated vaccine was generated by Dr. Ashley Fink and Tanvi Potluri in our lab by infecting MDCK cells at an MOI of 0.01 and collecting the infected cell supernatant at 72 hours post-infection. The infected cell supernatants were centrifuged at 500xg for 10 min and the virus containing supernatant was collected. The virus containing supernatant was inactivated by adding 0.05% β-propiolactone at 4°C overnight followed by incubation at 37°C for 2 h to allow the β-propiolactone to break down into non-toxic byproducts (Schaecher, Mackenzie, & Pekosz, 2007). The inactivated virion byproducts were purified by ultracentrifugation over a 20% sucrose cushion in a Beckman SW28TI rotor at 26,000rpm for 1 h at 4°C. The purified virus containing pellet was then resuspended in 1XPBS and a BCA assay (Pierce) was used to quantify protein concentration. Virus inactivation was confirmed following virus inactivation and virus purification by performing a $TCID_{50}$ assay.

Experimental protocols.

1. **Infection/Pathogenesis**

   Adult (7-18 weeks old) male C57BL/6NJ and $TLR7^{-/y}$ mice weighing 25.2 ± 2.3 g and female C57BL/6NJ and $TLR7^{-/-}$ mice weighing 19.5 ± 1.3 g were anesthetized with a ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and inoculated intranasally with 30 μL of a low dose of the ma2009 IAV (0.04 50% mouse lethal dose [MLD_{50}]). Body mass, rectal temperature, and mortality were monitored for 21 days or animals were euthanized for tissue collection at specified time points post-infection. Mice were euthanized with an intraperitoneal (ip) overdose of ketamine-xylazine. Tissues were collected at 0, 3, 5, or 9 days post-infection for virus titration and histopathology. Tissues collected for virus titration were snap frozen, stored at -80°C until processing, and then homogenized in .5 mL of DMEM. Homogenized samples were centrifuged and supernatants were collected and stored at -80°C.

2. Vaccine/Challenge

Adult (7-12 weeks old) male C57BL/6NJ and *Tlr7*$^{-/y}$ mice weighing 24.6 ± 1.5 g and female C57BL/6NJ and *Tlr7*$^{-/-}$ mice weighing 19.1 ± 2.0 g were vaccinated intramuscularly (i.m.) in the hind leg with 20 μg inactivated virus in 40 μL PBS at day 0 and boosted with the same dose, 20 μg inactivated virus in 40 μL PBS at day 21(Bodewes et al., 2010; Budimir, de Haan, Meijerhof, Gostick, et al., 2013; Budimir, de Haan, Meijerhof, Waijer, et al., 2013; Geeraedts et al., 2008). Serum samples were collected by non-terminal submandibular blood sampling using the 5 mm Golden Rod at 14 days, 28 days, and 35 days post-vaccination to measure antibody and neutralizing antibody titers. Vaccination was confirmed by performing an anti-IAV IgG ELISA assay using serum collected at 14 days to ensure the highest serum dilution OD value was above the average OD of the negative controls; all mice met these criteria.

At 42 weeks post-vaccination, mice were anesthetized with a ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and inoculated intranasally with 30 μL of a lethal dose of ma2009dv (32 MLD_{50}s). Body mass, rectal temperature, and mortality were monitored for 21 days or animals were euthanized for tissue collection at specified time points post-lethal challenge. Mice were euthanized by cervical dislocation after administering an intraperitoneal (ip) overdose of ketamine-xylazine. Tissues were collected at 1, 3, or 5 days post-challenge for virus titration and histopathology. Tissues collected for virus titration were snap frozen, stored at -80°C until processing, and then homogenized in .5 mL of DMEM. Homogenized samples were centrifuged, and supernatants were collected and stored at -80°C.

Histology

The right lungs from euthanized mice were inflated at constant pressure, fixed in Z-fix fixative (Anatech) for at least 48 h, embedded in paraffin, cut into 5-μm sections, and mounted on glass slides. Tissue sections were stained with hematoxylin and eosin (H&E) and used to evaluated lung inflammation. Histopathological scoring for lungs post-infection was performed by single veterinary

pathologist blind to the experimental groups. Histopathological scoring for lungs post-challenge was performed by a single graduate researcher blind to the experimental groups under the supervision of a veterinary pathologist. Three representative sections of each lung were scored and then the three scores were averaged. Scores were given for vascular inflammation, bronchial inflammation, and edema on a scale ranging from 0 to 3 (1-mild, 2-moderate, 3-severe) with 0.5 increments.

Virus titration

MDCK cells were plated in 96-well plates, grown to confluence, and infected with serial 10-fold dilutions of lung homogenates in infection media in replicates of 6. The cells were incubated for 6 days at 32°C, stained with naphthol blue black (Sigma-Alderich) and cytopathic effect (CPE) was scored visually. The Reed-Muench method was used to calculate the tissue culture infectious dose that caused CPE in 50% of a monolayer of MDCK cells ($TCID_{50}$).

Virus neutralization assay

Serially diluted serum was mixed with 100 $TCID_{50}$s of virus (ma2009 IAV) for 1 h at room temperature and used to infect quadruplicate wells of confluent MDCK cells for 24 h at 37°C. After incubating for 16 to 18 h, the inoculum was removed, the cells were washed 1 x with PBS (with calcium and magnesium), and fresh medium was added. Next the cells were incubated for 6 days at 32°C and then fixed with 4% formaldehyde. CPE was scored following staining with naphthol blue black. The titer was calculated as the highest serum dilution that eliminated virus CPE in 2 out of 4 wells per dilution. A sample was considered below the limits of detection if there was no neutralization at the 1:4 dilution of serum.

Antibody Enzyme-linked immunosorbent assays (ELISAs)

Enzyme-linked immunosorbent assay (ELISA) plates (Microlon 96-well high binding plates; Greiner Bio-One) were coated with 100 ng of purified virus overnight at 4°C in carbonate puffer (pH 9.6). The plates were washed 4 times with PBST (1 x PBS plus 0.1% Tween 20 [Sigma]) and blocked for at least

1 h at 37°C with 10% dry milk powder in 1 x PBST. The plates were washed 4 times and serially diluted serum with a starting dilution of 1:500 was added to the plates for 1 h at 37°C. Anti-mouse horseradish peroxidase (HRP) conjugated secondary IgG (1:250, Thermo), anti-mouse HRP IgG2c (1:20,000, Thermo), or anti-mouse HRP IgG1 (1:6,000, Thermo) was added, and the plates were incubated for 1 h at 37°C. The plates were washed 4 times with PBST, and the reactions were developed with 3,3'5,5'-tetramethylbenzidine (TMB; BD Biosciences) and stopped using 1 N hydrochloric acid (HCl). The absorbance at 450 nm of the plates was read on a plate reader. To determine the sample ELISA titer, a cutoff value was obtained by multiplying the average optical density (OD) values for the negative controls at each dilution by 3. The titer of the sample was calculated as the highest serum dilution with an OD value above the cutoff.

Influenza antibody avidity index

ELISA plates were coated with 100 ng of purified virus (as described above) overnight at 4°C in carbonate puffer (pH 9.6). The plates were washed 4 times with PBST and blocked for at least 1 h at 37°C with 10% dry milk powder in 1 x PBST. The plates were washed 4 times again and serum samples were added to the wells at a 1:60 dilution for 1 h at 37°C. To measure antibody avidity, 4M ammonium thiocyanate (NH_4SCN, Sigma) or 1XPBS was added to the plates for exactly 15 minutes at room temperature. The pates were washed 8 times with PBST and anti-mouse HRP conjugated secondary IgG (1:250, Thermo) was added for 1 h at 37°C. The plates were washed 4 times with PBST, and the reactions were developed with 3,3'5,5'-tetramethylbenzidine (TMB; BD Biosciences) and stopped using 1 N hydrochloric acid (HCl). The absorbance at 450 nm of the plates was read on a plate reader. The antibody avidity index was determined by normalizing the NH_4SCN treated absorbance values to the corresponding 1XPBS (untreated) values for each sample in duplicate.

Statistical analyses

Morbidity data was analyzed with a one-way ANOVA followed by Tukey post-hoc testing using the greatest body mass loss observed over the entire time-period as the dependent variable. Antibody titers,

virus titers, and histopathological data were analyzed using one-way ANOVA followed by Tukey post-hoc testing. Mean differences were considered statistically significant if p was <0.05. Graphs were constructed in GraphPad Prism and data analyses were run in STATA and GraphPad Prism.

RESULTS

The absence of TLR7 does not alter sex differences in outcome of influenza infection

We first examined the contribution of TLR7 signaling pathways to sex differences in host defense following IAV infection. Prior studies conducted in our lab have demonstrated that IAV infection results in greater body mass loss, used as a surrogate measure of clinical disease, among female C57BL/6CR mice as compared to males (Robinson et al., 2011). To test whether the absence of TLR7-dependent pathways modifies these sex differences in influenza-induced morbidity, we infected wild-type (WT) and *Tlr7* deficient mice intranasally (i.n.) with a sublethal dose of ma2009 IAV and analyzed body mass loss over 20 days following inoculation (Figure 1A). A one-way ANOVA was conducted to determine if body mass loss was different for male and female mice in the presence and absence of *Tlr7*. Mice were classified into four groups: WT males (n=5), *Tlr7*$^{-/y}$ males (n=9), WT females (n=5), and *Tlr7*$^{-/-}$ females (n=7) and data was collected from two independent experiments. There was a statistically significant difference between groups as determined by one-way ANOVA ($F(3,22)=11.70$, $p=.0001$). As expected, a Tukey post-hoc test revealed that body mass loss was statistically greater in female WT mice as compared to male WT mice (-11.3 ± 3.3 % body mass loss, $p=.011$) (Figure 1B). Surprisingly, female *Tlr7*$^{-/-}$ mice also lost significantly greater body mass loss as compared to male *Tlr7*$^{-/y}$ mice (-12.4 ± 2.6% body mass loss, $p<0.001$) and there was no statistically significant differences between male WT mice and male *Tlr7*$^{-/y}$ mice (.88 ± 2.9% body mass loss, $p=0.99$), or female WT mice

and female *Tlr7*$^{-/-}$ mice (-.24 ± 3.0% body mass loss, p=1.0). Thus, the absence of TLR7 did not significantly affect sex differences in body mass loss following IAV infection.

To further investigate the role of TLR7-mediated host defense pathways in protecting male and female mice over the course of a sub-lethal IAV infection, we collected and analyzed lung samples from infected mice at 0, 3, 5, and 9 days post-inoculation (Figure 1A). A one-way ANOVA was conducted to determine if viral titers were different for male and female mice in the presence and absence of TLR7 at 3, 5, and 9 days post-inoculation (dpi). Mice were again classified into four groups for each day: WT males (3 dpi n=5, 5 dpi n=5, 9 dpi n=10), *Tlr7*$^{-/y}$ males (3 dpi n=9, 5 dpi n=5, 9 dpi n=11), WT females (3 dpi n=5, 5 dpi n=5, 9 dpi n=10), and *Tlr7*$^{-/-}$ females (3 dpi n=5, 5 dpi n=5, 9 dpi n=9) and data was collected from 4 independent experiments.. There was no statistically significant difference between groups as determined by a one-way ANOVA at 3 dpi ($F(3,20)=0.71$, p=.56) or 5 dpi ($F(3,16)=1.24$, p=.33), but there was a statistically significant difference at 9 dpi ($F(3,36)=6.78$, p=0.001) (Figure 1C). Tukey post-hoc test at 3, 5, and 9 dpi revealed that the average viral titer was statistically lower in female WT mice than male WT mice at 9 dpi (-1.46 ± .40 $\log_{10}$ $TCID_{50}$/mL, p=0.003). In contrast there was no statistically significant difference between the mean viral titer of *Tlr7* deficient female and male mice at 9 dpi (-.42 ± .40 $\log_{10}$ $TCID_{50}$/mL, p=.71). Furthermore, there was no statistically significant difference in the mean viral titers of females in the presence or absence of TLR7 at 9 dpi (.99 ± .41 $\log_{10}$ $TCID_{50}$/mL, p=.081), while male *Tlr7* deficient mice had statistically significant lower viral titers than male WT mice (-2.01 ± .39 $\log_{10}$ $TCID_{50}$/mL, p<0.001). Therefore, sex differences in viral titers observed among WT mice were eliminated as a result of *Tlr7* deficiency in male mice.

The reduction in viral titers observed in male *Tlr7*$^{-/y}$ mice could possibly be explained by increased inflammatory responses in the lung, we therefore examined pulmonary inflammation in tissue sections of the lungs collected at 3, 5, and 9 dpi. Male and female mice deficient in *Tlr7* on average showed similar levels of pulmonary inflammation as WT mice, with no significant difference in the average cumulative inflammation score between any of the treatment groups as determined by

a one-way ANOVA at 3 dpi ($F(3,13)=0.65$, $p=.60$), 5 dpi ($F(3,16)=2.12$, $p=.14$), and 9 dpi ($F(3,16)=0.74$, $p=0.54$) (Figure 1D).

TLR7 mediates sex differences in quantity and quality of antibodies produced following influenza vaccination

We next examined the contribution of TLR7-dependent signaling to sex differences in response to vaccination. *Tlr7* is expressed in B cells which are responsible for producing antibodies that establish long-lived adaptive immunity and protection. To better understand the possible role of TLR7-dependent signaling in antibody production following vaccination, we collected serum and measured anti-IAV antibody responses at 28 and 35 days post-vaccination (dpv) (Figure 2A). A one-way ANOVA was conducted to determine if antibody titers were different for male and female mice in the presence and absence of TLR7 at 28 and 35 dpv. Mice were again classified into four groups for each day: WT males (28 and 35 dpv n=14), *Tlr7*$^{-/y}$ males (28 and 35 dpv n=14), WT females (28 and 35 dpv n=14), and *Tlr7*$^{-/-}$ females (28 and 35 dpv n=11), and data was collected from 3 independent experiments. There was a statistically significant difference between the average anti-IAV IgG titer of the treatments groups at 28 dpv ($F(3,49)=7.58$, $p<.001$) and at 35 dpv ($F(3,49)=4.88$, $p=.0048$). Tukey post-hoc testing at 28 and 35 dpv revealed that the average anti-IAV IgG titer of WT females was significantly higher than WT males at 35 dpv ($-154{,}500 \pm 42{,}522$, $p=.0025$), but there was no statistically significant difference at 28 dpv ($-97{,}429 \pm 42{,}522$, $p=.11$) (Figure 2B). In contrast, female *Tlr7* deficient mice mounted similar anti-IAV IgG titers as male *Tlr7* deficient mice with no statistically significant difference in the average anti-IAV IgG titers at 28 dpv ($-13{,}737 \pm 45{,}328$, $p=.99$) and 35 dpv ($-8{,}185 \pm 45{,}328$, $p=1.0$). Additionally, female WT mice on average had significantly higher anti-IAV IgG titers than female *Tlr7* deficient mice at 35 dpv ($-178{,}351 \pm 45{,}328$, $p<.001$), but not at 28 dpv ($-98{,}156 \pm 45{,}328$, $p=.14$). Male WT mice on average had similar anti-IAV IgG titers as male *Tlr7* deficient mice at 28 dpv ($14{,}464 \pm 42{,}522$, $p=.99$), and 35 dpv ($-32036 \pm 42{,}522$, $p=.88$).

The lack of sex differences in anti-IAV IgG titers among *Tlr7* deficient mice does not rule out sex differences in the ability of antibodies to bind or neutralize virus particles. To exclude the possibility of compensatory sex differences in antibody quality, we analyzed immune sera at 28 and 35 dpv using neutralization (Figure 2C) and avidity (Figure 2D) assays. Data was collected from 3 independent experiments. A one-way ANOVA analysis was conducted to determine if mean neutralizing antibody titers and mean antibody avidity indices were different among the four treatment groups at 28 and 35 dpv. There were significant differences in the average neutralizing antibody titers at 28 dpv ($F(3,49)=5.63$, $p=0.002$) and 35 dpv ($F(3,49)=3.25$, $p=0.029$) (Figure 2C). Additionally there were significant differences in the average antibody avidity index at 35 dpv ($F(3,48)=13.31$, $p=<.001$) (Figure 2D). Tukey post-hoc testing at 28 and 35 dpv revealed that on average female WT mice had anti-IAV neutralizing antibody titers significantly higher than male WT mice at 28 dpv ($-3{,}500 \pm 962.1$, $p=0.0025$) and antibody avidity indices significantly higher than that of male WT mice at 35 dpv ($-.16 \pm 0.048$, $p=0.007$). Among *Tlr7* deficient mice there was no sex difference in average anti-IAV neutralizing antibody titer at 28 dpv (-226.7 ± 982.6, $p=1.0$) or avidity at 35 dpv ($-.77 \pm .047$, $p=.37$). Furthermore, female WT mice on average had anti-IAV neutralizing antibody significantly greater than female *Tlr7* deficient mice at 28 dpv (3810 ± 999.9, $p=.0014$) and greater avidity indices than female *Tlr7* deficient mice at 35 dpv ($.21 \pm .049$, $p<0.001$). In contrast male WT mice on average had similar anti-IAV neutralizing titers to male *Tlr7* deficient mice at 28 dpv (537 ± 944.1, $p=.94$), but avidity indexes that were on average significantly higher than male *Tlr7* deficient mice at 35 dpv ($0.13 \pm .046$, $p=.038$) (Figure 2C, D).

IgG isotype switching to IgG2a/c has been shown to play a critical role in influenza vaccine efficacy and protection (Baldridge & Buchmeier, 1992; Coutelier, 1987; Heer et al., 2007; Kaminski et al., 1986) and previous studies suggest that TLR7 may play a role in this process (Heer et al., 2007; Jeisy-Scott et al., 2012). To further detail possible sex differences caused by TLR7-depedent signaling in the antibody response to vaccination, we examined isotype switching in immune sera at 35 dpv. This timepoint was chosen due to statistically significant sex differences observed in anti-IAV IgG titer

among WT mice. Data was collected from 2 independent experiments. One-way ANOVA analysis of isotypes revealed that at 35 dpv the anti-IAV IgG2c antibody titers are significantly different among the four treatment groups ($F(3,30)=16.76$, $p<0.001$). Tukey post-hoc analysis revelated that female WT mice on average had significantly higher anti-IAV IgG2c antibody titers than male WT mice (-150,056 ± 48,083 , p=.020), while female *Tlr7* deficient mice had comparable IgG2c antibody titers as male *Tlr7* deficient mice (-833.3 ± 48,083, p>.99) (Figure 2E). At the same time point, anti-IAV IgG1 antibody titers did not differ between the four treatment groups ($F(3,29)=2.20$, $p=0.11$) (Figure 2F). However, consistent with prior studies (Heer et al., 2007) male and female *Tlr7* deficient mice together had significantly different IgG1 titers than male and female WT mice($F(1,31)=8.66$, $p=0.006$). On average *Tlr7* deficient mice had IgG1 titers significantly greater than WT mice (8923.7 ± 3032, $p=0.006$). Together these results suggest that in female mice, TLR7-dependent signaling has an important role in the production IgG2c antibodies and neutralizing antibodies with high avidity.

Tlr7 deficiency eliminates sex differences in protection following challenge with an IAV drift variant virus

Having demonstrated that female *Tlr7* deficient mice generate lower vaccine-induced antibody titers and lower quality antibody following vaccination than female WT mice, which eliminates sex differences in antibody responses, we then examined whether *Tlr7* deficiency also eliminates sex differences in vaccine-induced protection following challenge. Previous studies conducted in our lab have shown that vaccinated female WT mice are better protected following challenge with a drift-variant virus, losing less weight than vaccinated male mice (Dr. Ashley Fink, unpublished data). To evaluate the possible role of TLR7-dependent signaling in vaccine-induced protection, we compared body mass loss, a surrogate measure of clinical disease, of vaccinated male and female mice in the presence and absence of *Tlr7* following a lethal challenge with ma2009dv IAV (Figure 3A). One-way ANOVA analyses were conducted to determine if average percent body mass loss was different for male and female mice in the presence and absence of *Tlr7*. Mice were classified into four groups: WT males (n=9), $Tlr7^{-/y}$ males (n=9), WT females (n=8), and $Tlr7^{-/-}$ females (n=8) and data was collected

from 2 independent experiments. As expected, average percent weight loss was significantly different among all four groups ($F(3,30)=9.14$, $p<0.001$). Furthermore, Tukey post-hoc testing revealed that vaccinated male WT mice on average lost more body mass than vaccinated female WT mice, however this difference was not statistically significant (1.59 ± .92 % body mass loss, p=.33) (Figure 3B). Vaccinated male *Tlr7* deficient mice on average lost significantly more body mass than vaccinated female *Tlr7* deficient mice (2.57 ± .92 % body mass loss, p=.04). This was surprising because no sex differences were observed in the quantity or quality of anti-IAV antibody produced prior to challenge. Additionally, vaccinated male *Tlr7* deficient mice on average lost significantly more body mass than vaccinated male WT mice (-3.10 ± .89 % body mass loss, p<.01) and female *Tlr7* deficient on average lost more body mass than vaccinated female WT mice, however the difference between female mice was not significant (-2.12 ± .95 % body mass loss, p=.14).

To further detail the possible role of TLR7-dependent signaling in vaccine-induced protection against IAV, we next assessed the host's ability to clear virus from the lungs. This is the most important parameter for vaccine efficacy. To measure viral clearance, we collected lung samples from vaccinated mice at 1, 3, and 5 days post-challenge (dpc) and analyzed viral load (Figure 3A). Mice were again classified into four groups for each day: WT males (1 dpc n=9, 3 dpc n=10, 5 dpc n=10), *Tlr7*$^{-/y}$ males (1 dpc n=10, 3 dpc n=10, 5 dpc n=13), WT females (1 dpc n=10, 3 dpc n=9, 5 dpc n=10), and *Tlr7*$^{-/-}$ females (1 dpc n=8, 3 dpc n=9, 5 dpc n=9) and data was collected from 6 independent experiments. A one-way ANOVA analysis was used to determine if there were significant differences in the mean viral titer of the four treatment groups at 1, 3, and 5 dpc. Significant differences were found in average viral titer at 1 dpc ($F(3,33)=3.38$, $p<0.05$), 3 dpc ($F(3,34)=3.48$, $p<0.05$), and 5 dpc ($F(3,38)=22.15$, $p<0.001$). As seen previously in our lab (Dr. Ashley Fink, data unpublished), Tukey post-hoc testing revealed the average viral titer was significantly lower in vaccinated female WT mice as compared to vaccinated male WT mice at 3 dpc (-1.39 ± 0.57 $\log_{10}$ $TCID_{50}$/mL, $p<0.05$) and 5 dpc (-1.03 ± 0.42 $\log_{10}$ $TCID_{50}$/mL, $p<0.05$). In contrast, vaccinated female *Tlr7* deficient mice had viral titers comparable to vaccinated male *Tlr7* deficient mice at 3 dpc (-1.40 ± .71 $\log_{10}$ $TCID_{50}$/mL, p=0.06) and

5 dpc (-1.01 ± 0.53 $\log_{10}$ $TCID_{50}$/mL, p=0.083). Additionally, vaccinated male *Tlr7* deficient mice on average had similar viral titers to vaccinated male WT mice at 3 dpc (0.52 ± 0.71 $\log_{10}$ $TCID_{50}$/mL, p<0.47) and significantly higher viral titers than vaccinated male WT mice at 5 dpc (2.29 ± 0.47 $\log_{10}$ $TCID_{50}$/mL, p<0.001). Female *Tlr7* deficient also on average had similar viral titers to vaccinated female WT mice at 3 dpc (0.51 ± 0.57 $\log_{10}$ $TCID_{50}$/mL, p=0.39), and significantly higher viral titers than vaccinated female WT mice at 5 dpc (2.31 ± 0.48 $\log_{10}$ $TCID_{50}$/mL, p<0.05). Together these data suggest that female mice are better protected by vaccines than male mice and this protection is eliminated in the absence of TLR7-dependent signaling.

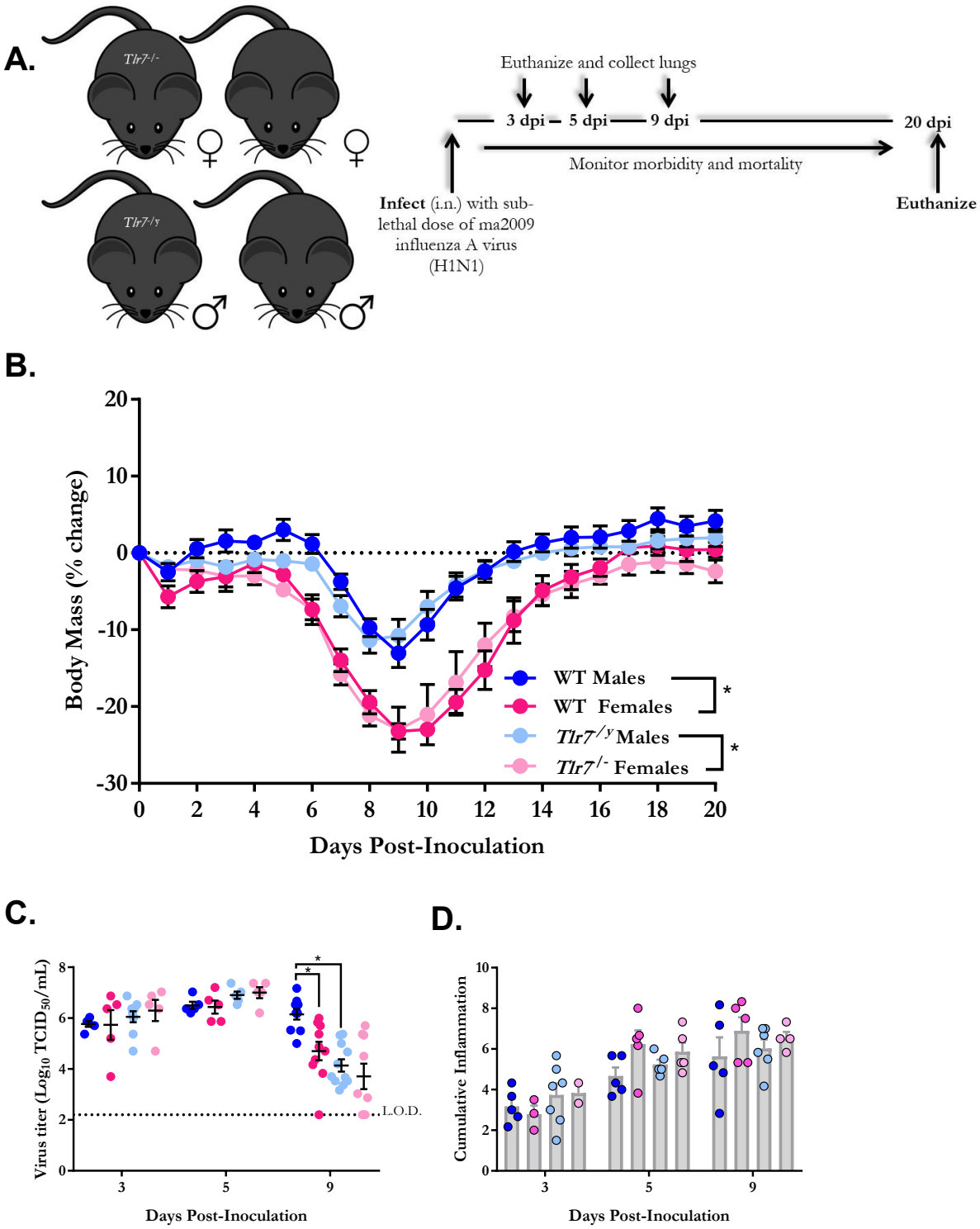

Figure 1. Toll-like receptor 7 did not affect sex differences in clinical disease following influenza infection. (A) Infection scheme. C57BL/6NJ mice were inoculated intranasally (i.n.) with mouse-adapted (ma) 2009 H1N1 influenza A virus. **(B)** Following infection, body weight was recorded daily as a surrogate measure of morbidity. Body mass loss curve shows results of n=5-9 from two independent infection experiments. **(C)** The viral load in lung tissue was determined from lungs collected at 3, 5, and 9 days post-inoculation (dpi) by $TCID_{50}$ assay. Results are presented from 4 independent experiments with n=5-11/treatment group/time point. **(D)** Inflammation in lung tissue collected at 3, 5, and 9 dpi was quantified based on a 0-3 scale of perivascular, peribronchiolar and alveolar inflammation. * = $p < 0.05$,. L.O.D. = limit of detection.

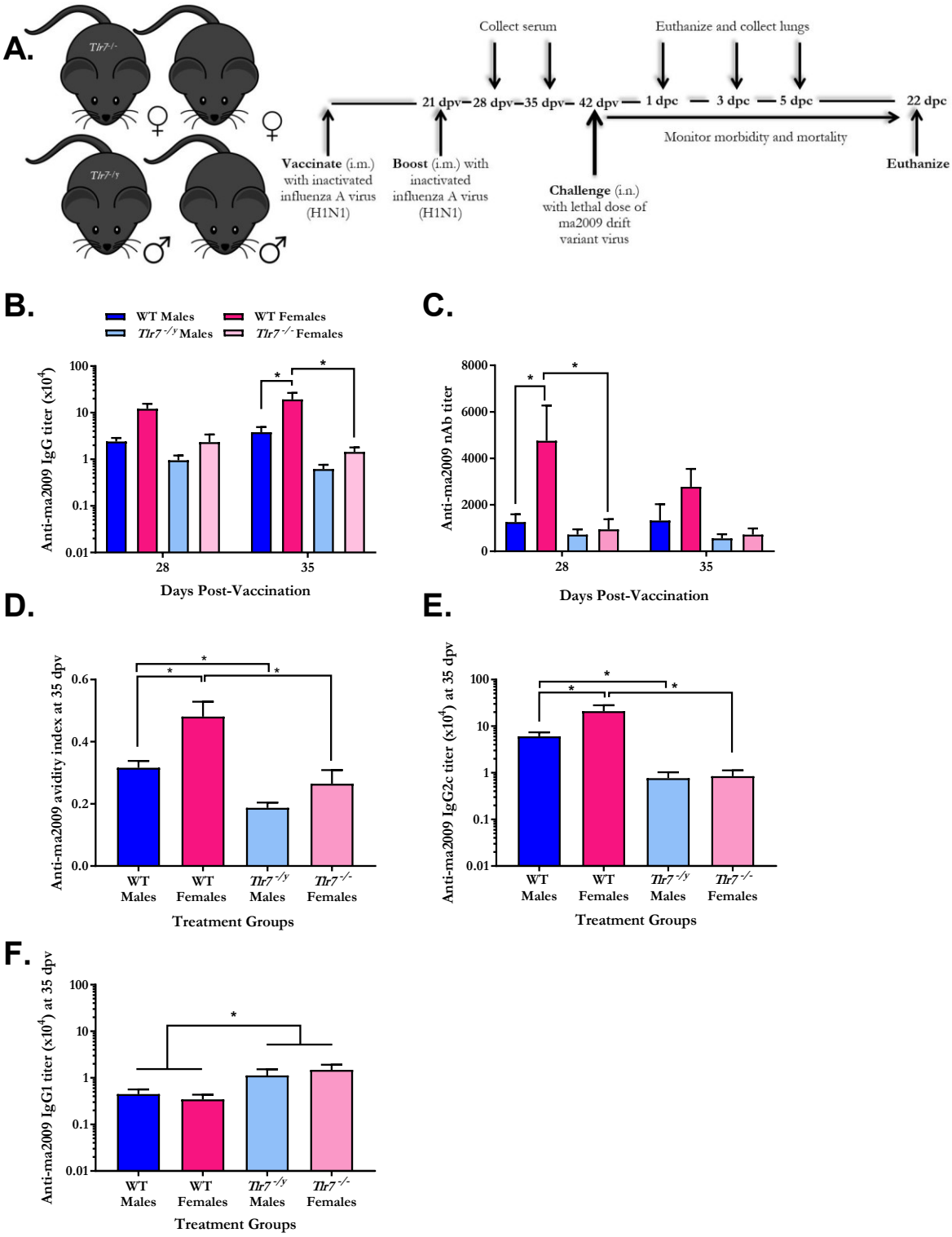

Figure 2. Sex differences in antibody titers and quality are eliminated in the absence of TLR7. **(A)** Vaccination and challenge scheme. C57BL6/NJ mice (n=7-14/treatment group) were vaccinated intramuscularly (i.m.) with 20μg inactivated mouse-adapted (ma) influenza A virus (H1N1) in 40 μL PBS and boosted with the same dose at 21 days post-vaccination (dpv). **(B)** Serum IgG antibody titers were measured at 28 and 35 dpv by ELISA assay. **(C)** Serum neutralizing antibody titers were measured at 28 and 35 dpv by neutralizing antibody assay. **(D)** Serum IgG antibody avidity was measured at 35 dpv by avidity ELISA assay. **(E)** Serum IgG2c antibody titers were measured at 35 dpv by ELISA assay. **(F)** Serum IgG1 antibody titers were measured at 35 dpv by ELISA assay. * = $p < 0.05$

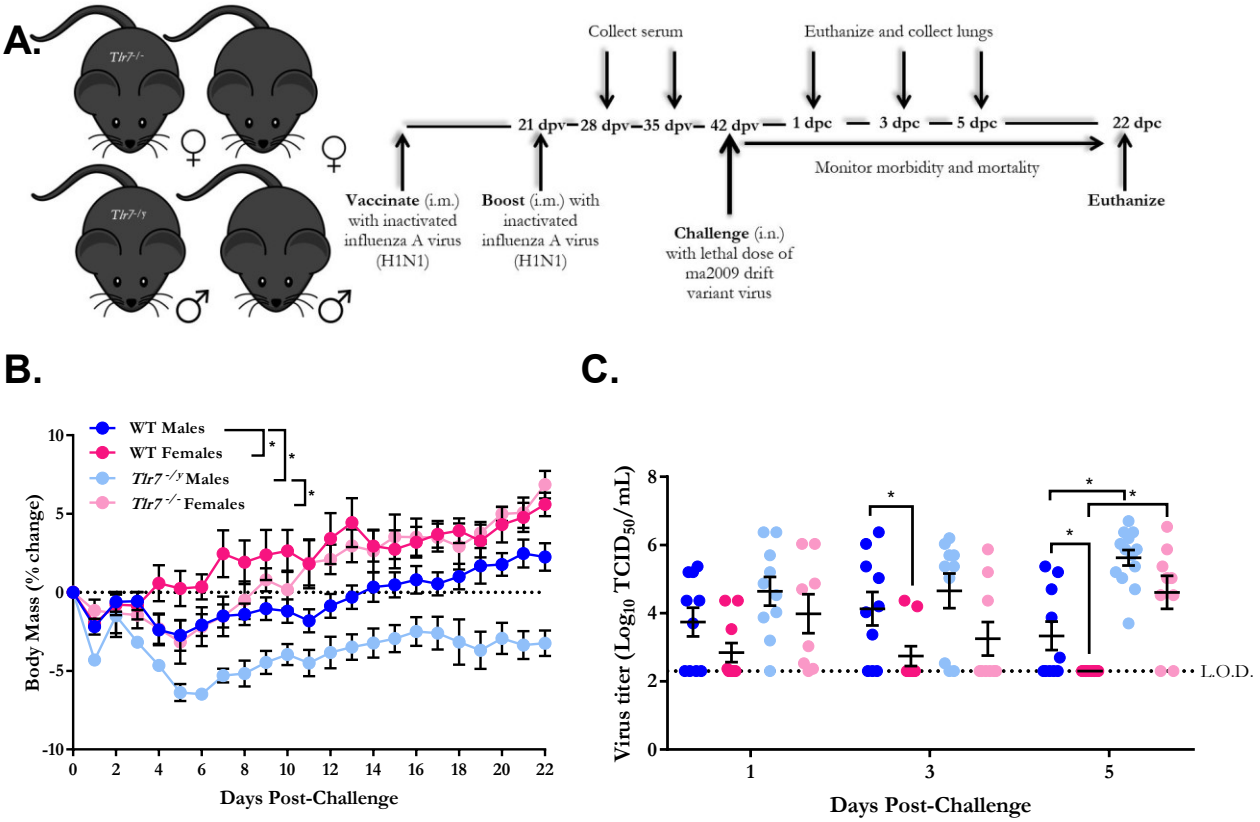

Figure 3. ***Tlr7*** **deficiency eliminates sex differences in protection following challenge with an IAV drift variant virus. (A)** Vaccination and challenge scheme. C57BL6/NJ mice (n=7-13/treatment group) were vaccinated intramuscularly (i.m.) with 20μg inactivated mouse-adapted (ma) influenza A virus (H1N1) in 40 μL PBS and boosted with the same dose at 21 days post-vaccination (dpv). At 42 dpv, vaccinated mice were challenged intranasally (i.n.) with a lethal dose of ma2009 drift variant virus. **(B)** Following challenge, body weight was recorded daily as a surrogate measure of morbitity. Weight loss curve shows results of n=7-10 from two independent experiments. **(C)** The viral load in lung tissue was determined from lungs collected at 1, 3, and 5 days post-challenge (dpc) by $TCID_{50}$ assay. * = $p < 0.05$, L.O.D. = limit of detection.

DISCUSSION

Several studies of humans and mice have shown that the severity of influenza virus infection and the efficacy of influenza virus vaccination significantly differs between males and females. Despite increasing evidence that TLR7, one of the PRRs that detects influenza viruses, escapes XIC in a significant proportion of female immune cells (Souyris et al., 2018), it's role in mediating sex differences in influenza infection and vaccine has not been studied. In the present study, we investigated the effects of TLR7-dependent signaling on sex differences in influenza pathogenesis, influenza vaccine response, and influenza vaccine-induced protection. We found that in C57NL/6NJ mice, TLR7-dependent signaling independently mediates sex differences in the antibody response to influenza vaccine and vaccine-induced protection but does not independently mediate sex differences in influenza related morbidity following infection of naïve mice. Following influenza vaccination, female mice produced higher antibody titers than males, but only in the presence of TLR7-dependent signaling. Decreased antibody titers following vaccination significantly affected the vaccine-induced protection of female *Tlr7*$^{-/-}$ mice upon challenge by increasing virus titers in the lungs.

TLR7 is thought to play a differential role during influenza infection versus immunization because of the different context in which viral RNA (vRNA) is recognized by PRRs in the two contexts. In the context of infection, vRNA can be recognized by a combination of at least three different families of PRRs over the course of IAV's life cycle in late endosomes or the cytosol, RIG-I, TLRs,

and NLRP3 (Ichinohe, Lee, Ogura, Flavell, & Iwasaki, 2009; Jeisy-Scott et al., 2012). Thus the absence of a unique weight-loss phenotype in TLR7 deficient mice in this study could be explained by redundancy in TLR7 function or by the sufficiency of multiple PRR pathways in the absence of TLR7 to facilitate the induction of the adaptive immune response (Jeisy-Scott et al., 2012; Trinchieri & Sher, 2007). It is of interest to note that sex differences in influenza induced morbidity were maintained even in the absence of TLR7, suggesting that either redundant PRRs or the combination of PRR pathways activated during influenza infection act in a sex-specific manner.

While there was no unique weight loss phenotype in TLR7 deficient mice following influenza infection, there were significant sex differences in the control of infectious viral titer. In this study, the absence of TLR7-dependent signaling in male mice, but not female mice, was associated with lower viral titers at 9 dpi. Decreased viral titers in *Tlr7* deficient mice have been found in previous studies as measured by nucleoprotein RNA-copies via qRT-PCR following infection with a more virulent IAV strain and in a study of mice also deficient in mitochondrial antiviral signaling (MAVS), part of the RIG-I pathway (Pang et al., 2013; Stegemann-Koniszewski et al., 2016). This finding is also consistent with other studies of knockout mice lacking a single TLR, which in most cases have either normal control of infection or more resistance to infection than knockout mice lacking an adaptor protein such as MyD88 (Akira, Uematsu, & Takeuchi, 2006; Fritz & Girardin, 2005; Trinchieri & Sher, 2007). Importantly, findings from this study make the novel contribution that TLR7-depedent signaling may play a sex-specific role in regulating viral replication in males. However, further investigation of sex differences in the activation of cytotoxic T cells which are associated with viral clearance and the role of sex hormones could provide more insight into the biological mechanism behind these findings.

Alternatively to influenza infection, in the context of immunization with an inactivated influenza vaccine, the inactivated viral particles are primarily recognized through the cell-intrinsic endocytic pathway by TLR7 (Jeisy-Scott et al., 2012). Therefore, endosomal TLR7 plays a critical role in activating the immune response during vaccination. Our findings support previous work which has shown that TLR7-dependent signaling in B cells is important for the induction of appropriate anti-

influenza humoral responses following vaccination with TLR7 deficient mice having increased IgG1 antibody and decreased IgG2c antibody titers (Heer et al., 2007; Jeisy-Scott et al., 2012). Furthermore, this study shows that TLR7-depedent signaling mediates sex differences in the quality of anti-influenza humoral response of C57BL/6NJ mice following influenza vaccination. Detailed analyses of the efficacy of different antibody isotypes have identified IgG2c as the key isotype for anti-influenza antibody-mediated effector functions in C57BL/6 mice (Baldridge & Buchmeier, 1992; Coutelier, 1987; Heer et al., 2007; Kaminski et al., 1986). Our findings, consistent with prior research conducted in our lab, show that female mice have higher IG2c antibody titers than male mice in response to influenza vaccination and these sex differences are eliminated in the absence of TLR7-dependent signaling; however, IgG1 antibody titers did not differ by sex in either treatment group. We also found that the absence of TLR7-depedent signaling eliminated sex differences in the avidity and neutralization potential of antibodies generated in response to vaccination.

Given the role of IgG2c antibodies in vaccine efficacy and the lack of sex differences observed in IgG2c antibody titer in *Tlr7* deficient mice, we hypothesized that male and female mice would be equally unprotected when challenged with a lethal dose of a ma2009dv IAV following vaccination. To our surprise female mice lost less body mass following a lethal challenge than male mice in the absence of TLR7-depedent signaling. Whereas male *Tlr7*$^{-/y}$ mice lost significantly more weight than male WT mice, female *Tlr7*$^{-/-}$ mice lost comparable amount of body weight to their WT counterpart. More importantly, differences in viral titer following challenge closely matched trends in antibody titer. There was a significant difference between female *Tlr7*$^{-/-}$ mice and female WT mice in viral titer at 5 dpc and no sex difference between *Tlr7* deficient mice at the same time point, as also seen in antibody titers following vaccination.

This study shows that TLR7-depedent signaling mediates sex differences in the antibody response to vaccine and viral clearance upon secondary infection with a drift variant virus. Additionally, differences in antibody titers were closely correlated with protection as measured through viral clearance following challenge. Persistent sex differences in morbidity following vaccination and

challenge in the absence of TLR7-dependent signaling or sex differences in antibody titer, may have been due to the robust nature of the female immune response. It is well established that female humans and mice have a stronger innate and adaptive immune response. Therefore, it is possible that more signaling pathways must be disrupted in order to disrupt body mass loss following lethal challenge in females or a decreased amount of antibody is still sufficient to confer protection in females. Deeper analysis of the adaptive immune response to vaccination and challenge in *Tlr7* deficient males and females would reveal sex differences independent of TLR7-depedent signaling.

From an evolutionary perspective, the ability of females to quickly generate a lot of antibody is critical for providing passive immunity to offspring through breastmilk. Newborns leave an almost germ-free intrauterine environment to enter a highly contaminated extrauterine state and antibodies in breastmilk provide protection from an abundance of potentially pathologic organisms that could lead to numerous infectious conditions. Furthermore, there is increasing evidence that antibodies in breastmilk may also play an important role in the development of the infant's microbiome and intestinal immune homeostasis (Walker & Iyengar, 2015). TLR7 may escape XIC in females to allow mothers to generate sufficient antibodies in breastmilk to protect their offspring from deadly pathogens. This would explain why females have the ability to produce more antibodies in the more modern context of vaccination. Interestingly B cell activating factor (BAFF) which is present in human breastmilk was recently shown to augment IgA2 production in TLR7 stimulated B cells isolated from human blood samples, however more data is needed to warrant further investigation of this hypothesis (den Hartog et al., 2018).

In summary, this study provides new evidence that TLR7-depedent signaling mediates sex differences in the production of antibodies following influenza vaccination and viral clearance following subsequent challenge. Future studies should investigate sex differences in different cell populations activated following vaccination to better understand how *Tlr7* mediates better protection in females following challenge. Additionally, these data support further investigation of the sex specific effects of using TLR7 adjuvants in vaccines which may only be effective for improving vaccine efficacy

in males. Lastly, sex differences must be considered in all future pre-clinical and clinical research of influenza.

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EDUCATION

MASTER OF SCIENCE, BIOCHEMISTRY AND MOLECULAR BIOLOGY May 2018
Johns Hopkins School of Public Health, Baltimore, MD
Thesis:

POST-BACCALAUREATE HEALTH PROFESSIONS PROGRAM May 2015
University of California Berkeley Extension, Berkeley, CA

BACHELOR OF ARTS, SOCIOLOGY May 2010
University of Redlands, Redlands CA

RESEARCH EXPERIENCE

GRADUATE RESEARCH ASSOCIATE November 2016 – June 2018
JHSPH, Department of Molecular Microbiology and Immunology Baltimore, MD

- Managed care and treatment of 40-100 mice, including injections and measurements;
- Quantitatively analyzed experimental data;
- Developed protocols for vaccine mouse model, including a new technique for non-terminal bleeding of mice.

PATIENT REGISTRY SPECIALIST April 2016 – August 2016
Pancreatic Cancer Action Network Manhattan Beach, CA

- Managed the development and testing of new registry features;
- Collaborated with clinic staff and patients to develop surveys on key research topics;
- Interviewed patients with pancreatic cancer about their experience.

MONITORING AND EVALUATION ASSISTANT January 2012 – January 2013
Tostan Dakar, Senegal

- Collaborated with program staff to improve the design of multiple Tostan programs;
- Researched, developed, and implemented a new procedure for processing surveys using the program CSPro;
- Co-led the development of monitoring and evaluation tools for the Child Protection Project;
- Drafted baseline, midterm, and final evaluation reports for donors;
- Led training sessions in computer skills and other technical areas.

CLINICAL TRIALS LABORATORY ASSISTANT June 2011-October 2011
San Francisco Department of Public Health HIV Research Section San Francisco, CA

- Monitored test results using an electronic clinical trial management system;
- Prepared clinical and laboratory supplies including detailed trial paperwork and blood collection tubes for multiple studies;
- Prepared, processed, and shipped time-sensitive clinical laboratory specimens;
- Conducted quality assurance activities.

PROFESSIONAL SERVICE

- Ad-hoc Reviewer: Journal of Immunology (2017)
- Member, Student Governing Board - Johns Hopkins University Student Outreach Resource Center, SOURCE (2016-2018)
- Volunteer – Thread (2016 – 2017)

GRANT RECEIVED

- Student Assembly Student Research Award. Received 2017 from Johns Hopkins School of Public Health, amount $1000.

STUDENT MENTORSHIP

- Julia Fountain (2018), Master's student in the Department of Biochemistry and Molecular Biology

RESEARCH SKILLS

- Animal models: C57BL/6 mice
- Laboratory: BSL-II
- Animal work: (i) experimental design and implementation for virus pathogenesis and vaccine efficacy studies; (ii) animal handling, sampling, vaccination, challenge, and euthanasia
- Antibody assays: ELISA, Neutralization, TCID50
- Histopathology: Tissue processing
- Mammalian cell culture: MDCK
- Statistical software: STATA, GraphPad Prism

PUBLICATIONS AND PRESENTATIONS

- Fink AL, **Engle K**, Klein SL. 2018. Biological sex affects vaccine efficacy and protection against influenza in mice. (Manuscript under review)
- Potluri T, **Engle K**, Fink AL, vomSteeg LG, Klein SL. 2017. Sex reporting in preclinical microbiological and immunologic research. mBio8:e01868-17
- **Engle K**. Biological sex affects vaccine efficacy and protection against influenza in mice. Organization for the Study of Sex Differences 12th Annual Meeting. May 2018.
- **Engle K**. Biological sex affects vaccine efficacy and protection against influenza in mice. Johns Hopkins Vaccine Initiative 10th Annual Vaccine Day. April 2018.
- **Engle K**. The role of TLR7 in mediating sex differences in response to influenza infection and vaccination. Biochemistry and Molecular Biology Department Retreat. April 2018.
- **Engle K**. Sex-specific dependence on toll-like receptor 7 for antibody response to influenza vaccination. Johns Hopkins School of Public Health Delta Omega Poster Competition. March 2018.